Identification, characterization, and quantitative expression analysis of rainbow trout myostatin-1a and myostatin-1b genes

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Abstract

Myostatin is a potent negative regulator of skeletal muscle growth. Although several cDNA clones have been characterized in different vertebrates, the genomic organization and bioactivity of non-mammalian homologs have not. The intron/exon organization and promoter subsequence analysis of two rainbow trout myostatin genes, *rtMSTN-1a* and *rtMSTN-1b* (formerly 1 and 2 respectively), as well as a quantitative assessment of their embryonic, larval, and adult tissue expression profiles are reported herein. Each gene was similarly organized into three exons of 490, 368, and 1600 bp for *MSTN-1a* and 486, 386, and 1419 bp for *MSTN-1b*. Comparative mapping of coding regions from several vertebrate myostatin genes revealed a common organization between species, including conserved pre-mRNA splice sites; the first among the fishes and the second across all vertebrate species. *In silico* subsequence analysis of the promoter regions identified E-boxes and other putative myogenic response elements. However, the number and diversity of elements were considerably less than those found in mammalian promoters or in the recently characterized zebrafish *MSTN-2* gene. A quantitative analysis of the embryonic expression profile for both genes indicates that rtMSTN-1a expression is consistently greater than that of rtMSTN-1b and neither gene is significantly expressed throughout gastrulation. Expression of both steadily increases fourfold during somitogenesis and subsides as this period ends. After eyeing, however, rtMSTN-1a mRNA levels ultimately rise 20-fold by day 49 and peak before hatching and yolk sac absorption (YSA). Levels of rtMSTN-1b rise similarly, but do not peak before YSA. An analysis of adult (2-year-old fish) tissue expression indicates that both transcripts are present in most tissues although levels are highest in brain, testes, eyes, muscle, and surprisingly spleen. These studies suggest that strong selective pressures have preserved the genomic organization of myostatin genes throughout evolution. However, the different expression profiles and putative promoter elements in fishes versus mammals suggests that limitations in myostatin function may have evolved recently.

Journal of Endocrinology (2006) **190,** 879–888

Introduction

Muscle growth results from the proliferation of myoblasts and their subsequent differentiation into muscle fibers. This process is regulated *in vivo* through mechanisms that involve cell-to-cell interactions, cell-to-matrix interactions, and extracellular secreted factors including myostatin (also known as growth/differentiating factor (GDF)-8) (Lee 2004). This member of the transforming growth factor (TGF) β-superfamily is a potent negative regulator of skeletal muscle growth. Indeed, a myostatin-null phenotype in domestic mammals is characterized by extreme gains in muscle mass, commonly referred to as 'double muscling' (Kambadur *et al.* 1997, McPherron & Lee 1997). In addition, a 5′ splice site mutation in the first intron of the human myostatin gene has recently been reported in a child with extraordinary musculature (Schuelke *et al.* 2004). Increased muscle growth in all these models results from both muscle cell hyperplasia and hypertrophy as myostatin influences myosatellite cells directly (Thomas *et al.* 2000, Rios *et al.* 2001, 2002, Langley *et al.* 2002, 2004, McCroskery *et al.* 2003). These results together suggest that the biological functions of myostatin are conserved in all mammals, although they are yet to be described in other vertebrates.

A recent phylogenetic analysis of the entire myostatin/GDF-11 subfamily (Kerr *et al.* 2005) indicates that bony fish possess multiple myostatin genes and that a gene duplication event during early fish radiation (Amores *et al.* 1998, Postlethwait *et al.* 1998) produced two distinct myostatin clades: MSTN-1 and MSTN-2. A second duplication event within salmonids, likely resulting from tetraploidization, produced two subsequent divisions, one in each clade. This suggests that most, if not all, salmonids possess four distinct myostatin genes: two within the first clade (1a and 1b) and two in the second (2a and 2b). Both the previously identified rainbow trout cDNA clones, formerly

DOI: 10.1677/joe.1.06866
0022–0795/06/0190–879
Online version via http://www.endocrinology-journals.org

named *Tmyostatin*-1 and *Tmyostatin*-2, are actually MSTN-1 orthologs. They were, therefore, renamed rtMSTN-1a and rtMSTN-1b respectively, which reflects their true evolutionary relationship to other myostatin genes (Kerr *et al.* 2005).

Myostatin genes have been characterized in mice (McPherron *et al.* 1997), humans (Gonzalez-Cadavid *et al.* 1998), cattle (Jeanplong *et al.* 2001), and pigs (Stratil & Kopecny 1999). Although cDNA clones have been characterized in many diverse fish species (Ostbye *et al.* 2001, Roberts & Goetz 2001, Rodgers & Weber 2001, Rodgers *et al.* 2001, Rescan *et al.* 2001, Maccatrozzo *et al.* 2001*a*, Kocabas *et al.* 2002, Kerr *et al.* 2005), very few genes have been completely characterized. This is particularly disconcerting since bony fish, especially teleosts, represent the largest group of extant vertebrates and many of these species are commercially important. Therefore, a better understanding of the genomic sequence and organization of different fish myostatin genes, as well as species-specific expression patterns will significantly interest comparative and agricultural biologists alike. This information will be particularly important in the identification and cloning of *MSTN-2* genes from different salmonids and will help in distinguishing paralogs from orthologs.

The isolation and characterization of the *rtMSTN-1a* and *rtMSTN-1b* genes, including their respective promoter regions, are reported herein. We additionally report the quantitative assessment of the expression of each gene using detailed RNA panels generated from multiple stages of embryonic/larval development and different adult tissues. These studies indicate strong sequence conservation among all vertebrate myostatin genes. However, the expression patterns and putative promoter elements suggest that the function of myostatin in fish may not be as limited as in mammals.

Materials and Methods

Isolating genomic myostatin clones

Genomic DNA was extracted from rainbow trout (*Oncorhynchus mykiss*) fin clips. Briefly, 3 ml lysis buffer (30 mM Tris, 8 M urea, 4% w/v Chaps (pH 8·0) was added to 50 mg tissue and incubated overnight with proteinase K (20 mg/ml) at 60 °C. Three consecutive phenol:chloroform:isoamyl alcohol extractions were then performed and DNA quality was verified on a 1% agarose gel. Promoter regions were cloned using the Universal Genome walker kit (BD Biosciences, www.bdbiosciences.com) and the manufacturer's protocol. Briefly, genomic DNA was digested with the blunt-end restriction enzymes DraI, EcoRV, PvuII, and StuI, and subsequently ligated to the provided adaptor linkers. Nested PCR with 94 °C initial denaturation was then performed using gene-specific primers homologous to the known 5′-coding region of each gene (Table 1) and adaptor primers with the Advantage 2 PCR kit (BD Biosciences). The cycle parameters were as follows and used as default unless otherwise specified: an initial denaturation at 94 °C for 1 min, seven cycles of 94 °C for 30 s, and 72 °C for 3 min followed by 30 cycles of 94 °C for 30 s, and 67 °C for 3 min and a final extension at 67 °C for 4 min. The PCR products were subcloned into the Topo TA vector (Invitrogen, www.invitrogen.com) and sequenced in the university's genomic core facility. Putative-regulatory elements were identified by subsequence analysis using Matinspector software (Genomatrix, Inc., www.genomatix.de), which searches consensus sequences of known *cis*-regulatory sequences. The intron sequences were obtained by nested PCR using gene-specific primers for rtMSTN-1a or rtMSTN-1b coding regions and adaptor primers (Table 1) as follows: initial denaturation at 94 °C for 1 min followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 3 min, and a final extension at 72 °C for 3 min. The resulting amplicons were then cloned as described and sequenced. Flanking primers, specific to each myostatin gene, were then used to amplify and clone the complete genes using Pfu polymerase (Stratagene, www.stratagene.com) and the pCR4-blunt Topo vector (Invitrogen). Intron/exon boundaries were determined by aligning the cDNA sequences to their respective gDNA sequences using ALIGN X (VectorNTI, www.invitrogen.com).

The 3′ untranslated regions were isolated using a 3′ RACE (rapid amplification of cDNA ends) kit (Invitrogen, www.invitrogen.com). The total RNA from juvenile skeletal

Table 1 Primer sequences and annealing temperatures (°C)

Primer name	**Sequence** (5′–3′)	**Anealing temperatures**
MSTN-1a F	CTT CAC ATA TGC CAA TAC ATA TTA	60
MSTN-1a R	GCA ACC ATG AAA CTG AGA TAA A	60
MSTN-1b F	TTC ACG CAA ATA CGT ATT CAC	60
MSTN-1b R	GAT AAA TTA GAA CCT GCA TCA GAT TC	60
18s F	TGC GGC TTA ATT TGA CTC AAC A	60
18s R	CAA CTA AGA ACG GCC ATG CA	60
3′UTR 1b/1	AAC TCT GTA GTC CGC CTT CAC GCA	65
3′UTR 1a/1	AAC TCT GTA GTC CGC CTT CAC ATA	65
3′UTR 2	CAC CTG CAG AAG TAC CCC CAC ACC	65
Adaptor primer 1	GTA CTA CGA CTC ACT ATA GGG C	67
Adaptor primer 2	ACT ATA GGG CAC GCG TGG T	67

muscle was extracted using Trizol and reverse transcribed using Superscript reverse transcriptase, according to the manufacturer's protocols (Invitrogen). cDNA was then amplified by PCR using gene-specific forward primers (3′UTR1a/1 and 3′UTR1b/1, Table 1) and universal adaptor primers (UAP) provided in the kit. The PCR conditions were as follows: an initial denaturation at 94 °C for 1 min followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 2 min. Nested PCR was subsequently performed with the 3′UTR2 forward primer and the abridged universal adaptor primer (AUAP) provided in the kit. The resulting amplicons were subcloned and sequenced as described.

Embryonic and tissue collections

Rainbow trout were reared at the National Center for Cool and Cold Water Aquaculture, Kearneysville, WV, USA, according to the guidelines approved by the institutional animal care and use committee. An RNA panel was generated from 5000 pooled eggs from multiple females (Trout lodge, October 2004) that were fertilized by milt from two males. Following fertilization, eggs were incubated at approximately 13 °C throughout embryonic development. In addition to the unfertilized eggs (day 0), developing embryos were collected as whole egg samples daily for the first 14 days, every other day until hatch (day 24), and every third day, thereafter. Each sample contained 18 eggs or embryos or 9 post-hatched larvae that were pooled and several samples were collected at each time-point. Tissues were also removed from 2-year-old adult fish weighing approximately 2 kg. All samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Samples were first powdered using a liquid nitrogen-cooled Bessman Tissue Pulverizer (Spectrum Laboratories, www.spectropor.com) and total RNA was extracted using TRI-reagent with the high-salt solution modification to remove excess glycosylated proteins. The RNA was reconstituted in 20–50 µl nuclease-free water and treated with DNase (DNAse RQ-1; Promega, www.promega.com) to remove contaminating genomic DNA. Samples were then re-extracted with TRI-reagent and RNA quality was assessed by agarose gel electrophoresis.

RNA quantification using 'real-time' reverse transcriptase (RT)-PCR

The total RNA (2 µg) was reverse transcribed with 1 µg random primers (Promega) and 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT, Promega) in 40 µl. Subsequent real-time RT-PCR assays were conducted using the ABI Prism 7900HT Sequence Detection System (www.appliedbiosystems.com) and gene-specific primers (MSTN-1a F, MSTN-1a R, MSTN-1b F, and MSTN-1b R, Table 1). For each sample, 1 µl cDNA was combined with 7·5 µl of 2× SYBR Green PCR master mix (Applied Biosystems). For each reaction, 6 µl of this mixture was added to 9 µl primer mix containing 500 nM of each primer. The reactions were carried out as follows: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. The cycling reaction was followed by a dissociation curve to verify amplification of a single product and amplicons were also verified by DNA sequencing.

The relative standard curve method was employed to quantify gene expression. For each primer set, a serial dilution of a mixed tissue cDNA was used to construct a standard curve for each assay plate. The standard curve was constructed by plotting the threshold cycle (C_T) versus the natural log of input RNA (ng). This curve was then used to calculate the relative abundance of each transcript in each sample. Myostatin values were then normalized to those of 18s to control differences in RNA and cDNA loading. Each sample was run in triplicate on a single plate and each plate was run in duplicate. Assays were repeated with different samples and all data are presented as normalized gene expression.

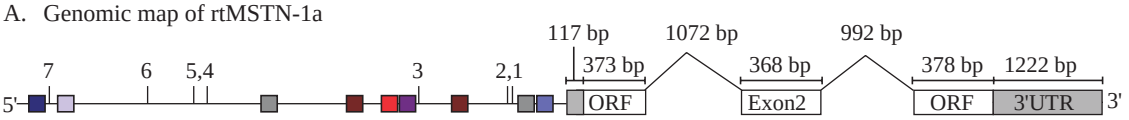
Results

Genomic organization and comparative mapping of rtMSTN-1a *and* rtMSTN-1b *genes*

Complete genomic clones for both *rtMSTN-1a* and *rtMSTN-1b* genes were isolated and sequenced (Figs 1 and 2). This includes, approximately, 2 kb upstream of each initiator. The annotated gene and promoter sequences were then deposited with GenBank and assigned the accession numbers DQ136028 and DQ138300 respectively. Each gene is organized into three exons of similar size with appropriate intron/exon splice sites, a pattern that is conserved with other fish species (Maccatrozzo *et al.* 2001*b*, Xu *et al.* 2003, Kerr *et al.* 2005) and mammals (McPherron *et al.* 1997, Gonzalez-Cadavid *et al.* 1998, Stratil & Kopecny 1999, Jeanplong *et al.* 2001). The three rtMSTN-1a exons are 490, 368, and 1600 bp in size respectively, and are separated by 1072 and 992 bp introns (Fig. 1A). The *rtMSTN-1b* gene is similarly organized with three exons of 486, 386, and 1419 bp with two, 564 and 778 bp introns intervening (Fig. 2A). The 3′ UTRs were also cloned by 3′ RACE using RNA from adult skeletal muscle and determined to be 1·2 kb in the rtMSTN-1a transcript and 1·1 kb in rtMSTN-1b. This is in contrast with the significantly shorter 3′ UTRs reported previously (Rescan *et al.* 2001). In addition, two polyadenylation signal sequences (AATAAA) were detected in each 3′UTR at −17 to −11 bp and −83 to −76 bp from the poly A site in rtMSTN-1a and −19 to −13 bp and −86 to −80 bp in rtMSTN-1b (Figs 1D and 2D).

The proteolytic processing sites and the entire bioactive domains for both myostatin proteins are entirely encoded within the third exons, which is also true for all previously characterized

A. Genomic map of rtMSTN-1a



B. rtMSTN-1a promoter

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-2284                    CTTT CCCCTACGGT TGATTTCAAT AAAGAAGTGT
-2250 CATACATCCA CACGGATTGT GAACCGAAAT ATGTGTGCCG CCTCTCCTTT
-2200 CTATTCAATC TCGTTATCGC CTTCAAACCG CTGCATATGC GTCCAATGAT
-2150 TTGAACTTGA AGCTTGGAAG GCAGCGATAG CATTGGTCGG TTTCTCTCTC
-2100 TGCACGAAAT GTGCACTACC TTACCAAACT GGGCACCATA CCACATTGTG
-2050 CAGTAATTTA ACTAAATGCT AAATCAATTT AAGGTCTTAA AGCCATGAAT
-2000 AGTCATACCT GGATGACTAC ACCTTCTTGC CATCATTCTA CTCGAGATTG
-1950 TCCATAGGTC ATCGTTGAAA TTGTTGCCTG TTTGACTACA CTTTCTAATG
-1900 GCGGCTGTAG CTTGTAGGAC GTAATTATAT TTTTAAAACA CTCTCAACAG
-1850 CAATTAGTAG CTGTGTCGAC TATGTCGTGG AGGTAAATTG TGCGCATACA
-1800 ATCAGGATAC TGATACTTGT ACAATATCAC CCTCTTGTGG CATGCTTGAC
-1750 TTCTCAGTGA TTTATTACAA TGTTTAAAGT AAGGAATATA TTCAGTTGTT
-1700 GCTATAAACT AATGCCATAG ATTATAATCC TGTCTCCCAG GCCTTTGCTG
-1650 GAAGTGTACC CCCGTGCAGC TTCCCTGTGT CTAAATTTTA GGCTACTACT
-1600 GAGTCTGCCA AGGGGTCTGG ACCATTATGG CACAGGAGGT ACGGTAGTGT
-1550 GCTTTCGCTT AAACCGCAGA GGTTTTATGA GTATTGATAG CAAGCCATAT
-1500 GTGCTCGGAT ATATTTCAAA GCAATTGTTG TCAGCTAAAA AAAAAATATA
-1450 TATATGACGA GTTCGTTCTT GACGAATGCG TAAATCTGTA AAACTCATGG
-1400 AGGAGTGTGC GTAAAACTGT ATAGTCCATA AGTGTAAATT AGTTAAAGTC
-1350 GTTTAGTATG TTTTCTTTCA CATAAGGGAT ATAGTCTCAA ATTCCATAAT
-1300 GATAATAGGC TATAAACCAT TGACATGACT CGTGACCAAG TCATTGTGTC
-1250 ATGCCATCCA TATTGCTGCG TAAAATGTAC ATAATTTAGA CGATTTGATT
-1200 GACTTTCATT AGAACCTATA TGTTACTTTT CATCTGTAAA TGAAAATAAT
-1150 ATTTCATAAC CTGTGTGTAA TGTTGCAACT GTAGTTGTCG ATAAGCCGCA
-1100 CGATGGCACT AAACGTCTAG ACAAAAAAGC TTTCGCAAAT CAGACACCCC
-1050 AAGGCAACAA TAACCAGTAG CCTATAGTTT AGTACAGTAT AGTAGGCCTA
-1000 CATTACAGGC CATTTCGCTA TATATTTGGA ATGGTATCTA TCCTATAGCC
-950  TATTGATTAA TACATTTTGC CTTTGGAGAG TAGGTTTGGA GACATGTAGG
-900  TCTACTTCAT GTAGCCTATA GGTCTACATA GACTAAATAT TGGTTCCTGT
-850  CATATTTGTA AATAATTGAC CTTACATTTT CAAGAGAGAT TCAGAAAGAT
-800  AGGCAAGCTG ATCATATAAC TAACTGATTG TAAAAGAAGT GGTCATAACT
-750  CAGATTAATT TGATCCCTGT AGCTTTGATG GGATGCAGGG GCGGACTGGC
-700  CATCTGGCAT TCCCGACAAA TGTCTGAAGG GTTGGTAAAT TCTTTGCCTA
-650  GAGGGCCTGT CTAACTTTTT TTATTTATTT TTTTTTGCGC AAAATGATAA
-600  TTATCTGGCT AATAATGGTG GCCTCAATTA ATAAAATGGG CTGGTGTGGG
-550  GGCCTTGATG AAACATTTTT AGTTGTGGGT GCCTCAAGGG GGAAAATGTG
-500  CCATTGTGTT ATAAATGCCA GAACCGATTT CTGGTTCCAG TCCACCCCTG
-450  GTGTGATGGG ATAGGCTGAA TAAAACAGGT TTCTCAGTGG CATTCAGATG
-400  TTGACAGGCT GTGGCTGACT TAAGTTCATG CAATAAATCA ATAACCATAT
-350  CCGAAATGTT TATTTGACCA TTTGCAGTAC TGTCTTGTCT GATTGATAGT
-300  GTAGTTGTCT CAAAACTATT CCAAGCCTTG CCATTGCGTA CCTCTTTAAG
-250  GTTTGACAGA AAAATACAGT TCATGCCTGT CTGTTAAATT CATTGTTGCC
-200  AGCCCAACCA ATCATAGATT TTGACGACAC AAAAGAGAGG CCAAAGTTGC
-150  AATATAAAAA GGGCTGCCGA ATTAAAGTAT GACACCTATC AGTGTAGAGC
-100  CTGATTTCAA ACACAGGCAA CTCTGTAGTC CGCCTTCACA TATGCCAATA
 -50  CATATTACAT TTGGGATTCA ATTTTTATAG CAAACTCCGC ACCTTAGATA
```

C. Promoter elements of rtMSTN-1a

Name	Location
Comp1	–236 to –256, –1190 to –1210
HAND2/E12	–694 to –708
MEF2	–477 to –499, –772 to –794
MusIn	–2161 to –2184
SRF	–2257 to –2281
TATAbox	–143 to –147
TEF-1	–683 to –699

D. 3'UTR of rtMSTN-1a

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TGAGCGAGAG CTCTGCCGGT GAGGGGGAGG GGCTCAGCCA GGGTCTCCC
CCCTGGACTT TGGGACAGAT CCATCCACCA CTACCAGTGC TTTCTGCAG
AACACGGTGC AATAGAGCCA GAATAGCGGC TAAAGAAATG CCCGTTCAT
TCGCTGAGCA GCGCTTCAAC CACGGACATG TCTCGTTCAT TTTTTTTCT
TCATTTTTGT TTTCTTCCCT CCTCCAATCA CATGTTCGCA GCCACAATG
GCCTAAAACC ACATGGATGT AGGAACACAA CGCCTGTTCG ACTTGGGAG
TGGACGTAGT AGCCTAAAGT ACGCCACATG TTCCCACAAA ACTGTTAAA
TGTCACACGC TCCGTCCAGT TATTCAAACA TACAGACATG GATGGACAC
GCATACACCA GACCTGGGTT CAAATAGTAT TTGTTTTTCT TTCAAATAC
TTTGACTGTT TGATGGAGAC AGGTGGGGTT TGCACTTTGG GGAATAATC
CATTGATTCC ATTGCACCAG GCAAGCACAG CTTAAGTACT GGAAAGAAA
ACAAATCATA TTTCAACCCA GGTCAGAAAC ATAGGTGCAC ACACACACA
CACACAGACT AATTTCCCCC TTAAGATTTT ACAGCTACGC TTACCTATC
GGTGTGATAA TCATATAAGC AATATTTGAG AGTGAAACCG GGAATCCTC
AACGACTTTT GAAAGGGCTT GGAAAACTAT AGCTGAGGTC TCAGTCTGA
ACTGGCCTTC ATGCATAATG CCATAAACAC ACACAGCAAC AGTGCATTC
TTATTCAACT TTTAATCATA GCTTTACCAC CATTCAACTG CCTGAACTG
CCCTACTCAC TTGAATTATT CTTATTGTAA ATCAACATTA CTGGACAGG
AGGACTTGAA CCGGAGGCAC AATGAATGCA GTCTACACAT TGAGATGTG
TTTAAGACAG ATAAATATAT TTTGAAAAGT ATGAATGTTA AAACCACGT
TTAAACTCTG TCTTACAAAT AACACAGTTT GCACTATGGC AAACCAATA
GAAAGAATTG GTTGCTACAA AAGTTGTAAA AACTGATTTT GATACGTTT
GCTAATTTGT ATTGTATACA TATGCCATTG TTTCCATTAG CAGTTGCCT
TTCTACACCA CTGTTAATAA ATGTATAAGA CCACAAACTA GCAAGAAAA
CAGTACAAAT GCGACTCTCT ACCTGTTAAT CAAATAAAGG TGCTTCCTT
AAAAAAAAAA AAAAAA
```

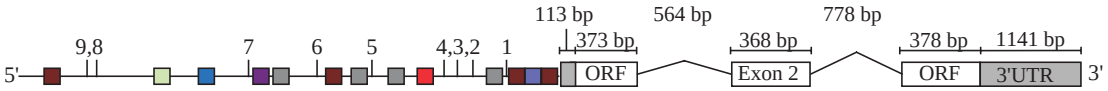
Figure 1 Genomic structure and organization of the *rtMSTN-1a* gene. (A) Map of *rtMSTN-1a* gene and putative myogenic *cis*-regulatory elements within the promoter region. Exons are boxed with the open reading frame (ORF) in white and untranslated regions (UTR) in gray. Each individual *cis* element is placed relative to its position within the promoter region and color-coded as indicated in C. Putative E-boxes are numbered. (B) Sequence of the promoter region with color-coded *cis* elements. Boxed are consensus sequences (CAN(T/A)TG) for E-boxes. Nucleotide position corresponds to the initiator. (C) Key to the color-coded-promoter elements in A and B, and their corresponding positions. (D) Sequence of the 3′ untranslated region. The newly identified sequence is underlined and two potential polyadenylation signal sequences are boxed.

vertebrate genes (Fig. 3). Comparative mapping of coding regions revealed a common organization between species, including conserved pre-mRNA splice sites. Codons flanking the first splice sites (3′ end of exon 1 and 5′ end of exon 2) are highly conserved among the fishes, while the second site (3′ of exon 2 and 5′ of exon 3) is conserved in fish and mammals (Fig. 3). Amino acid consensus sequences were, therefore, identified by comparative sequence analysis of intron/exon-coding junctions. These include MAT(E/K)|PXXI for the first junction and (G/E)(E/D)GL|XPFΦ for the second (X = any amino acid; Φ = hydrophobic, likely L, I, or M). Indeed, multiple sequence alignments with myostatin proteins from different vertebrates, both previously published (Rodgers & Weber 2001) and repeated with newly discovered clones (data not shown), indicate that these motifs are highly conserved, the first in fishes and the second in all vertebrates.

In silico *analysis of rtMSTN-1a and rtMSTN-1b promoters*

Subsequent analysis of the 2 kb promoter regions upstream of each gene using *MatInspector* software identified several putative muscle-specific transcription factor binding sites or *cis*-regulatory elements. These include Comp1 (cooperates with myogenic proteins 1), HAND2 (heart, autonomic nervous system, neural crest derivative 2), MEF 2 (myocyte enhancer factor 2), MusIn (muscle initiator), SRF (serum response factor), and TEF-1 (transcriptional enhancer factor 1) binding sites in the rtMSTN-1a promoter (Fig. 1).

A. Genomic map of rtMSTN-1b



B. rtMSTN-1b promoter

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-2425                                    ATCAT GGGAAGCGAC CCTTGAAACC
-2400 CCCTCCCCAC CTTTAACTAT AACCCACCTT TATTGAACTA ATAAAGCAT
-2350 ATGTGGAAGA ATGTCACTAT TCAAATGTGA ACATAAAAGG TCCAGGAGAA
-2300 ATACTAGCCC GGATGGAGGA AGGGCGAAAT AAATGGTTGT CCGCGTTGTA
-2250 AATAGGCTTC TAAGAATTCT TACATGAGCG TATTTCTGTT CAATATGTTT
-2200 AGAGGTAGCG TCTAGTCCAT TTCATACACA GTTACCAGAA AAATAGGTAC
-2150 ATGGAATATT GCTGAAGCGT GGGATAGGCA GCCTACCTTC AAAAAGACTC
-2100 TTGGAAAAGT AGGACTGGAT GGGTTTCCCT CCATGAATAT CAAATTCATC
-2050 CCGTAGGAAA ACGCATCGTT TATCTTGAAC GACACGTTTC AATAAAGAAG
-2000 TCTCATACAT CCACACGGTT TGTGAACCGA AATCACCAAA CGTGCCGCCT
-1950 CTCGGACTGT GTCTACTCCG TCTCCTTATC AAACCGCCGA ATATGCGTCC
-1900 AAGGCTTGAA CATGAAACTT GGAAGGCAGC GACCACATTG GTCAGGTTTC
-1850 TCTCTCAGCA CGAAATTTCC ACCAAGGTAC CAAACTGGAC ATTCAATGTT
-1800 CCACCATACC ACATTGTGCA GTAATCCCAC TAAATACAAC ATCGATTTAA
-1750 TTAAACTCTC ACATCCTGGA ATAGTCGGAC ATGGAGGACT ATACCTTCTT
-1700 CTTGCCATCA TTCTACTGGA GATTCTACAT AAATCATCGT TGAAAGTGTT
-1650 GCCTGTTTGA CTCAACGTTC TGAAGGCAGT TAGTAAACTG CAGAACGGGT
-1600 TGTGGAGATA AATTGTGCGC GTTCACACGG GTTACTGTTA CTTGTAGAAT
-1550 ATCACCCTCT TGTGGCGCAT GGTGGCTCCA TGCTTGACTC CCCAGTGACT
-1500 ATTGAAATGT TTAAAGTAAG GAATACAGTC AGTTGTTTCT ATAAGCTATC
-1450 TACCTATAGA TTACATTCCT GTCTCTTGCC TTTGATGGAC GTGTACCCAC
-1400 GATTAGCTTC CTTGTATCAA AGTGTTCTAA GGCTACTCAT GCGCCTGTCG
-1350 AGAGGTCTGG ATCATTATGG CACGAGGTAT ATTAGCGAGC TTTCCCAATA
-1300 GCCACTGCAG TCGCACTGTG TCAGATTTTC AGTTATGAGT TATCGGATAT
-1250 GTTCAAAACC AATTGGTGTC ATTTAAAAAA ATATATATGA CGCATTCATA
-1200 AAACAGTAAA ACTCATTGGA CGAGTGTGCG TAAAACGGTA TAATGTACAT
-1150 TAGTTAAATT CGTTTATGTT TTTGTAAATA CAGTATGGTA TATTTTCTTT
-1100 CACATAAGGG TTCCAGTCTC AAATTCGATA ACGACAATAG GCTATAATTA
-1050 AACCATTCAT TGACCTAACA TAGTGCCATG CCATTCATAT TGCTGCGTAA
-1000 AATGTGCATA AAGTGGGCAA TTTGATTGAC TCTTGTGAGC CATACACATT
 -950 TTCTAAATGC CGGTTGCGTT GATCTAAATT ATAATGCATA TGTTACTGTT
 -900 TATTTGTGAA GGAAAATAAT ATCTCATAAC CTGTGCGTAA TTTTGCAACT
 -850 GTTGTTGATT AACCGCATGA TGGCACTAAA CGTCTGGACT AAAAAGCTTT
 -800 CGTAAATAGT AAGCGCAAAC ACCTCAAGAC AACAATATCC AGTAGCCTAT
 -750 AGATTAATAC AGTATCGTAG GCCTACAGAA GAGCCCATTT CGCCATAAAT
 -700 GTTCAATGCA ATAGTATCTA TCCTATAGCC TATTGACAAA AGTATTTAGC
 -650 ATTTGGAGAC AAGTTTAGAA AGAAGTAGGT CTACTCCATG TAGCCTATAT
 -600 CAATATAGAC TAAAGAGTGG TTCCTGTCAT ATTTGTCAAT TAATATACCT
 -550 TGCATTTTCG AGAGAAACAG ATTTAGAGAG TTAGGCAAAC TGGTCATATA
 -500 AACTAACTGA TCACAGGGGA AGGGAAGTCA TAACACCGAT TCATTTGAAC
 -450 CCTGTTGCGT TGATGGGATG AGCGGAAAAA ACGCCTTTTC TCAGTGGCGT
 -400 TCCTATGGTG ACAGGCTTGC GGATTTAAGT TTATGCAATT AATCAATATA
 -350 ACCGCATCCG TAATTTGATT TGACCATTTG CAGAACTGTC TTGTCTTTGA
 -300 TTGGTAGTGT AGTTGTCTCA AAACTATTCC GAACCTTGCC ATACCTCTTT
 -250 TAAGGGTTGA CAGAAAAATA CAGTTGATGT CTGTCCGTTA AATTCATTGT
 -200 TGCCAGCCCA ACCAATCGTC GATTTTGACG ACACAAAAGA GAGGCCAAAG
 -150 TTGCAGTATA AAAAGGGCTG ACGAATTAAA GTATGCCACC TATCAGTGTA
 -100 GAGCCTGATT TTAAACACAG GCAACTCTGT AGTCCGCCTT CACGCAAATA
  -50 CGTATTCACT TTTGGATTTT TTTTTTATAG CAAACTCCGC ACCTTAGATA
```

C. Promoter elements of rtMSTN-1b

Comp1	–232 to –252, –830 to –850 –963 to –983, –1403 to –1423
Hand2/E12	–694 to –708
MEF2	–77 to –99, –128 to –150 –1044 to –1066, –2373 to –2395
MEF3	–1850 to –1862
MuscleTATA	–1660 to –1676
TATABox	–139 to –144
TEF-1	–1427 to –1443

D. 3'UTR of rtMSTN-1b

```
TGAGCGAGAG TTCTGCTGGG GAAGGGGAGG GGCTTAGCCA GGGTCTCCAC
CCTGGACTTT GGGACAGATC CATCCATCAC TACCAGTGCT TTCTGCAGAA
CACAGTGCAA TAGAGCCACA ATAGCGGCTA AAGAAACACC TGTCCATTCG
CTGAGCCGGC TTCAACCACG GACATGTCTC GTTCGTTTTT TTCATTTTCA
TTTTCTTCTC TCCTCCAATC ACATGTTCGC CCCACAATGG CCTAAAGTCA
CAAGGATGTA GGAACACAAT GCCTGTTGGA CTTGACATGG ACACACACCA
GACCTAGGTT CAAATAGTAT TTGTTTTTTT TCTTCAAATA CTTTAATGGA
GAAGGGTGGG GTTTGCACTT TGGGGAATAA TCCATTGGTT CCATTGAACC
AGGCAAGCTC AATCAAGTGC AGCTTAAGTA TTGGAAATAT AACAAATCCT
TTTTAAACCC AGGTCTGACA CACATAGACT AATCCCCCAA CCCCCAAGCT
TTTACAGCGT ACGTTTAACT ATCGGTGTGA TAATCATATA AGCAATTTTT
GAGAGTGAAA CCGGGAATCC TCAAGGACTT TTTAAAGGGC TTGGAAAACT
ATAGCTGAGG ACTCAGTCTG AACTGGCCTT CATGCACAAT GCCATAAACA
CCATATACTC ACACAGCAAC AGTGCATTAT TATTCAACTT TTAATCATAG
CTTTACCACC ATTCAACTGC CTGAACTGCC CTACTCACTT GAATTATTCT
TATTGTAAAT CACCATTACT GGACAGGAGG ACTTGAACCG GAGGCACAAT
GAAAGCAGTC TACACATTGA GATGTGTTTA AGACAGATAA ATATATTTTG
AAAAGTATGA ATGTTAAAAC CACGTTTAAA CTCTGTCTTA CAAATAACAC
AGTTTGCACT ATGGCAAACC AATTGAAAGA ATTGGTTGCT ACAAAAGTTG
TAAAAACTGA TTTTGATACG TTTGCTAATT TGTATTGTAT ACATATGCCA
TGGTTTCCTT TAGCAGTTGC CTTTTTACAC CACAGTTAAT AAATGTATAA
GGCCACAAAC CAGCAAGAAA ACAGTACAAA TGCGGCTATA TAACCTGTTA
ATCAAATAAA GGTGCTTGCT TATAAAAAAA AAAAAAAAAA AAAA
```

Figure 2 Genomic structure and organization of the *rtMSTN-1b* gene. (A) Map of *rtMSTN-1b* gene and putative myogenic *cis*-regulatory elements within the promoter region. Exons are boxed with the open reading frame (ORF) in white and untranslated regions (UTR) in gray. Each individual *cis* element is placed relative to its position within the promoter region and color-coded as indicated in C. Putative E-boxes are numbered. (B) Sequence of the promoter region with color-coded *cis* elements. Boxed are consensus sequences (CAN(T/A)TG) for E-boxes. Nucleotide position corresponds to the initiator. (C) Key to the color-coded promoter elements in A and B, and their corresponding positions. (D) Sequence of the 3′ untranslated region. The newly identified sequence is underlined and two potential polyadenylation signal sequences are boxed.

The rtMSTN-1b promoter contained all these putative elements except for the MusIn site and additionally contained a MEF 3 site (Fig. 2). Each promoter also contained an appropriately placed TATA box and several putative E-boxes, while a muscle TATA box was also found in the rtMSTN-1b promoter. A comparative analysis of all cloned myostatin gene promoters from fish revealed features common to all or most promoters and some features unique to a particular promoter as well (Fig. 4). Every promoter contained several E-box motifs and all, but the brook trout (bt) MSTN-1b promoter contained multiple Comp 1 sites and TATA boxes in close proximity to the transcription start sites. Studies with mammalian promoters indicate that MEF2, GRE (glucocorticoid-response element), and MyoD (myogenic differentiation factor) binding sites regulate myostatin promoter transactivation (Ma *et al.* 2001, Spiller *et al.* 2002, Forbes *et al.* 2006). These sites were also identified in the fish promoters. All, but the btMSTN-1b promoter contained multiple MEF2 sites and many were located within the first 500 bp. A GRE was only identified in the btMSTN-1b and zebrafish (zf) MSTN-2 promoters, while the latter additionally contained the only MyoD-binding site as well as two myogenin-binding sites. Roberts & Goetz (2003) previously identified a MyoD-binding site in the btMSTN-1b promoter, although this site was not identified in our analysis using the same, yet updated software package.

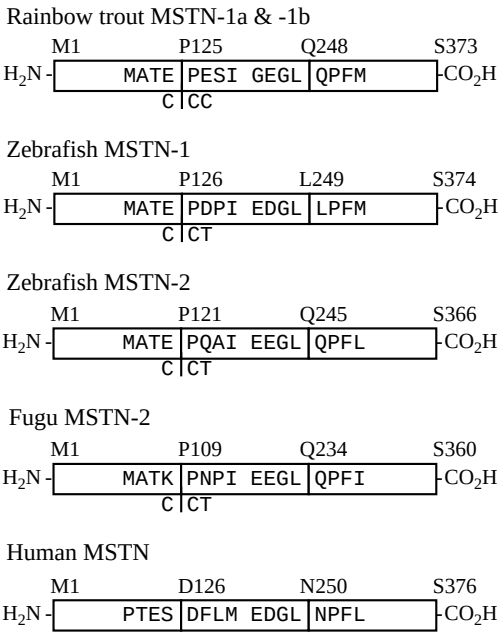

Figure 3 Comparative mapping of exon boundaries in different myostatin genes. All vertebrate myostatin genes cloned to date are organized into three exons. The three adjoining boxes for each protein represent the coding regions for each exon. Amino acid sequences coded by exon boundaries are shown inside the boxes. The first amino acid coded by each exon is shown above, as is the last residue of the third exons. In all fish genes, the codon of the proline residue located at the first exonic boundary is partially coded by the first and second exon as shown. Locations of the nucleotide splice sites for rtMSTN-1a and rtMSTN-1b are shown in Figs 1 and 2 and the sequences fit the known consensus.

Embryonic expression

A quantitative analysis indicated that both rtMSTN-1a and rtMSTN-1b were similarly expressed at low levels during the early stages of development. However, levels of both transcripts rose substantially after eyeing with rtMSTN-1a mRNA levels always greater than those of rtMSTN-1b (Fig. 5A). Expression of both, peaked and dropped immediately before hatching and then continued to rise thereafter. A similar peak in rtMSTN-1a expression also occurred just before yolk sac absorption, although this was not observed with rtMSTN-1b (Fig. 5C). A closer analysis of early developmental stages indicated that neither gene was significantly expressed during gastrulation, although expression of both steadily rose during somitogenesis peaking and then subsided at its end (Fig. 5B).

Adult tissue expression

Expression of both rtMSTN-1a and rtMSTN-1b was detected in every tissue sampled, including brain, pituitary gland, heart, ovary, testis, kidney, stomach, pyloric ceca, intestine, liver, pancreas, peripheral blood leukocytes, erythrocytes, spleen, gills, branchial arches, fins, skin, eyes, white and red skeletal muscles, and fat (Fig. 6). This includes, some tissues not known to express either myostatin (pituitary, stomach, pyloric ceca, pancreas, leukocytes, erythrocytes, spleen, brachial arches, fins, skin, eyes, and fat) and several others previously thought not to express rtMSTN-1b in particular, which has only been identified in brain and skeletal muscle (Rescan *et al.* 2001). Expression of both genes was highest (note log scale) in brain, testes, eyes, muscle, and surprisingly spleen. Individual tissue levels of both transcripts were similar in many tissues, but not all. Those of rtMSTN-1a were approximately 50-fold higher than rtMSTN-1b levels in fins and expression of the latter was almost 100-fold greater in leukocytes and gills, and 1000-fold in heart.

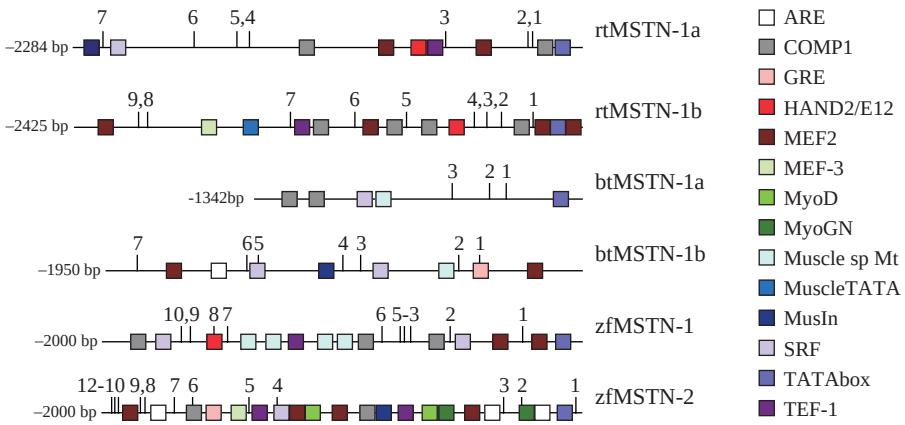

Figure 4 Comparative subsequence analysis of fish myostatin gene promoters. Promoter maps of rainbow trout myostatin rtMSTN-1a and rtMSTN-1b, brook trout btMSTN-1a, and btMSTN-1b and zebrafish zfMSTN-1 and zfMSTN-2 are shown with putative *cis*-regulatory elements boxed. Putative E-boxes are also indicated and numbered in ascending order starting from the transcription start site on the right. Each element is placed relative to its position within each promoter and is color-coded according to the key.

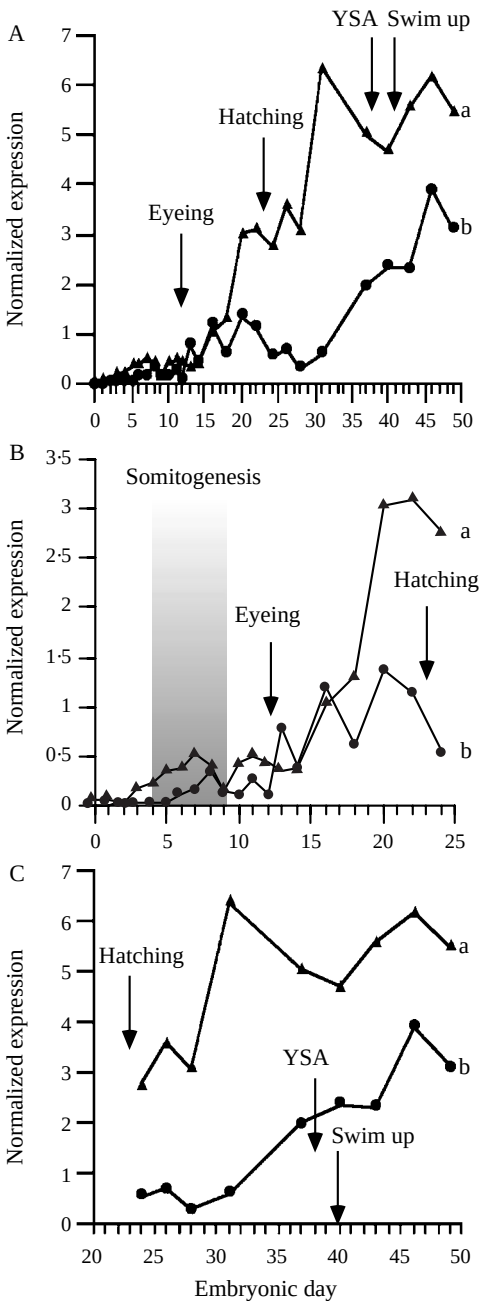

Figure 5 Developmental expression of rtMSTN-1a and rtMSTN-1b. A RNA panel was constructed from 5000 fertilized eggs and developing embryos sampled at the indicated days. Levels of rtMSTN-1a and rtMSTN-1b mRNA were quantified using gene-specific 'real-time' RT-PCR assays. Expression levels throughout the entire period from day 0 (unfertilized) to day 49 are shown in A, whereas more detailed assessments of early (days 0–24) and late (days 24–49) stages are shown in (B) and (C) respectively. Key developmental events are indicated by the labeled arrows and the gray box (YSA, yolk sac absorption). Each value represents a mean of three replicate measurements of a single pooled sample ($n=18$ embryos or 9 post-hatched larvae) at each time point. Assay variance was controlled as described in Materials and Methods.

Discussion

The genomic organization of both the rainbow trout *MSTN-1* genes (Figs 1 and 2) is highly similar to that of other homologs previously characterized in mammals and nearly identical to those in other fish. Indeed, exon boundaries and pre-mRNA splice sites are even conserved (Fig. 3), especially the second, which separates the coding region of the latency-associated peptide from the bioactive domain of mature myostatin. The amino acid identity of the mature bioactive domains of most fish and mammalian species is 88% (Rodgers & Weber 2001), indicating that both primary sequence and gene organization are highly conserved among vertebrates. Although a more comprehensive analysis of genes from divergent fish species and other vertebrate classes is needed to determine the degree of conservation across taxa, these data suggest that strong selective pressures are likely to be responsible and particularly important in preserving fidelity of the third exon. Teleosts commonly possess multiple copies of individual genes. This is a result of an early genome duplication event prior to the teleost radiation, but after the divergence of ray- and lobe-finned fishes (Amores *et al.* 1998, Postlethwait *et al.* 1998). A second duplication event specifically within the salmonids (Phillips & Rab 2001) gave rise to additional ('a' and 'b') myostatin paralogs within each MSTN-1 and MSTN-2 sister clade (Kerr *et al.* 2005), although none of these genes has been identified to date. Nevertheless, the high degree of genomic and sequence conservation shared among all myostatin genes and fish homologs (Fig. 3) should aid in their isolation and characterization.

Subsequent analysis of the rtMSTN-1a and rtMSTN-1b promoter regions identified several putative *cis*-regulatory elements that could contribute to the myogenic process. Some of these elements were also identified in the comparable promoters of brook trout (*Salvelinus fontinalis*) and zebrafish myostatin genes, including multiple MEF2 sites in each (Fig. 4). A putative MyoD site was also identified in the brook trout MSTN-1b promoter by Roberts & Goetz (2003), although this particular site was not identified in our analysis using the same, yet updated software. Among these fish genes, however, MyoD sites were identified in the zebrafish MSTN-2 promoter, which also contained far more putative myogenic elements than its counterpart (Kerr *et al.* 2005). Mammalian myostatin promoters contain E-boxes and other elements critical to the differentiation and maturation of skeletal muscle, including both MyoD- and MEF2-binding sites. Indeed, both these sites have been implicated in the regulation of myostatin-gene expression in different animal and cellular systems (Spiller *et al.* 2002, Salerno *et al.* 2004, Shyu *et al.* 2005). Expression of both *rtMSTN-1a* and *rtMSTN-1b* genes increases as somitogenesis progresses and rapidly decreases as it ends (Fig. 5B). This is consistent with increased transactivational activity of these and other myogenic-regulatory factors and myostatin's developmental expression

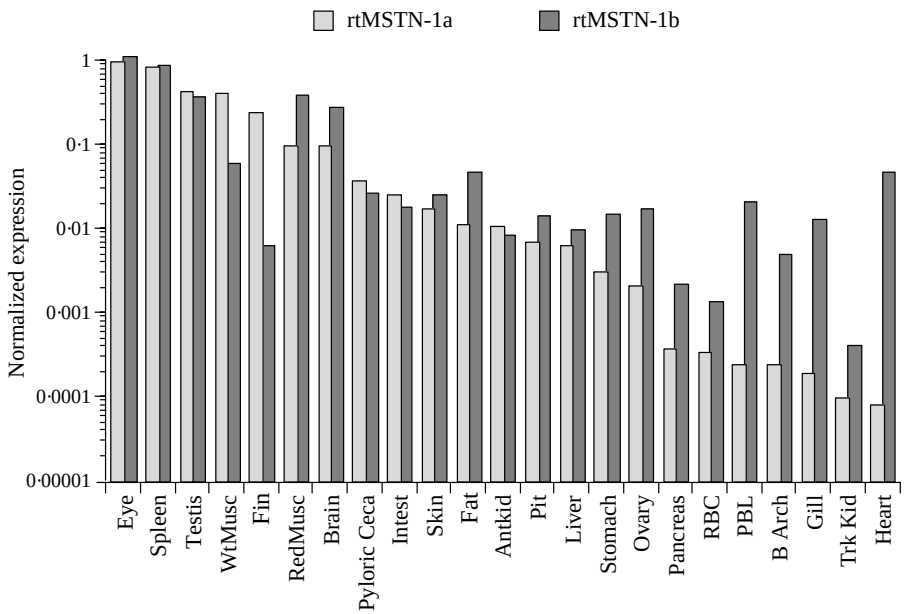

Figure 6 Adult tissue expression of rtMSTN-1a and rtMSTN-1b. Levels of mRNA for both genes were quantified using gene-specific 'real-time' RT-PCR assays and total RNA removed from the indicated tissues. Assays were performed on pooled tissue samples, but were run in triplicate and repeated twice. Mean values are shown. (Pit, pituitary gland; Ant kid, anterior/head kidney; Trk, trunk; Intest, whole intestine; PBL, peripheral blood lymphocytes; RBC, red blood cells; B Arch, branchial arch w\o gill fillaments; Wt Musc, white muscle).

profile in mouse embryos (McPherron *et al.* 1997). A functional assessment of promoter activity is needed to definitively determine whether these transcription factors regulate either *rtMSTN-1a* or *rtMSTN-1b* gene expression in developing skeletal muscle. The ubiquitous nature of MSTN-1 expression in fish, however, suggests that additional elements unrelated to myogenesis altogether may be active as well.

Former attempts to define the developmental and tissue-specific expression profiles of fish *MSTN-1* genes revealed a far more diverse expression pattern than, which occurs in mammals (Ostbye *et al.* 2001, Rescan *et al.* 2001, Rodgers *et al.* 2001, Maccatrozzo *et al.* 2001*b*, Kocabas *et al.* 2002, Roberts & Goetz 2003, Rodgers *et al.* 2003, Vianello *et al.* 2003, Johansen & Overturf 2005). These studies were still somewhat limited and mostly qualitative assessments. Rescan *et al.* reported that rtMSTN-1a mRNA levels were substantially higher than those of rtMSTN-1b in most adult tissues and at the three stages of development (eyeing, hatching, and free-swimming larvae). The one exception was adult brain where expression appeared equal for both genes. This study also indicated a very limited distribution of rtMSTN-1b expression, which was restricted to the brain and the skeletal muscle. By contrast, Ostbye *et al.* reported a much wider tissue distribution and apparently higher levels, in some tissues, of Atlantic salmon MSTN-1b expression. Both these studies used qualitative RT-PCR assays that do not account for primer efficiency and other aspects of non-quantitative PCR amplification and could have easily underestimated rtMSTN-1b expression. By contrast, our use of comprehensive RNA panels and a quantitative 'real-time' assay suggest that both *rtMSTN-1a* and *rtMSTN-1b* genes are expressed much earlier embryologically, specifically during somitogenesis, and in more adult tissues. Expression of both genes was detected in all tissues sampled and surprisingly high in spleen and eyes, which possibly indicates novel functional roles for myostatin in the growth and/or differentiation of immune and proliferative cells of the eye (Reh & Levine 1998). Johansen and Overturf also analyzed developmental expression of rtMSTN-1a and rtMSTN-1b using a quantitative RT-PCR assay. Although only a few developmental stages were sampled (eyed, hatched/sac present, and swim-up fry), their results also indicate that the expression of both genes rises substantially after eyeing and rtMSTN-1b mRNA levels are significantly higher than previously reported. Myostatin expression in mammals is first detected within the developing myotome (Kambadur *et al.* 1997, McPherron & Lee 1997), although former attempts to localize myostatin message in fish somites have produced mixed results (Xu *et al.* 2003, Amali *et al.* 2004, Kerr *et al.* 2005). Nevertheless, our results are the first to identify a temporal expression pattern in fish that is consistent with a functional role during the early stages of muscle development as levels of both rtMSTN-1a and rtMSTN-1b rise substantially throughout somitogenesis and begin to subside just before this developmental period ends.

The expression patterns described and the subsequence analysis of the different promoters further support a role for

both the *MSTN-1* genes during fish myogenesis, although the ubiquitous expression pattern in different adult tissues suggests that the functional role of cytokine is far more diverse than that in mammals. The presence of multiple fish genes that are differentially expressed throughout development and adult tissues also suggests that the precise role of a particular gene may vary between tissues. A better understanding of physiological factors that influence the expression of each gene and the transcriptional machinery involved will, therefore, help distinguish the potential divergent actions of myostatin in fish and mammals.

Acknowledgements

This work was supported by a grant from the United States Department of Agriculture (2004-34468-15199) to Buel D Rodgers. The authors wish to acknowledge Dr Carid Rexroad III, Kristy Anderson and Roseanna Athey for the assistance with embryonic sampling and tissue collection. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 15 March 2006
Received in final form 9 June 2006
Accepted 21 June 2006
Made available online as an Accepted Preprint
30 June 2006